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Patents ADP number *(if you know it)* — 7569734001

If the applicant is a corporate body, give the country/state of its incorporation — UK

4. **Title of the invention** — METHODS OF IMMUNOSUPPRESSION

5. **Name of your agent** *(if you have one)* — D YOUNG & CO

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D Young & Co.

D YOUNG & CO
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METHODS OF IMMUNOSUPPRESSION

Field of the Invention

The present invention relates to methods for preparing antigen presenting cells and regulatory T cells that can suppress the activity of other T cells. It also relates to the use of compositions capable of upregulating expression of an endogenous Notch ligand in such methods. These compositions, antigen presenting cells and regulatory T cells may be used in immunotherapy.

Background to the Invention

Immunological tolerance to self-antigens is vital to the proper functioning of the mammalian immune system. In addition to the deletion of self-reacting T cells in the thymus, active suppression mediated by regulatory T cells has recently been identified as an important mechanism for maintaining peripheral tolerance (WO98/20142). In autoimmune diseases such as multiple sclerosis, rheumatoid arthritis or diabetes, there is a failure of the proper regulation of tolerance. Improved treatment methods for re-establishing tolerance are desirable for autoimmune diseases. Similarly in allergic conditions and for transplantation of an organ or tissue from a donor individual, induction of tolerance to particular foreign antigens or profiles of foreign antigens is desirable.

It has recently been shown that it is possible to generate a class of regulatory T cells which are able to transmit antigen-specific tolerance to other T cells, a process termed infectious tolerance (WO 98/20142). The functional activity of these cells can be mimicked by over-expression of a Notch ligand protein on their cell surfaces. In particular, regulatory T cells can be generated by over-expression of a member of the Delta family of Notch ligand proteins. Delta expressing T cells specific to one antigenic epitope are also able to transfer tolerance to T cells recognising other epitopes on the same or related antigens, a phenomenon termed "epitope spreading".

WO98/20142 describes methods for generating regulatory T cells by either transfecting hybridoma T cells with a nucleic acid construct directing the expression of Delta or by

transfecting dendritic cells with a nucleic acid construct directing the expression of Serrate and incubating the dendritic cells with T cells.

Summary of the invention

The present invention identifies substances capable of upregulating expression of the endogenous genes encoding Notch ligands in antigen presenting cells and T cells. We believe that incubating APCs in the presence of these substances and a specific antigen produces APCs capable of inducing immunotolerance in T cells to the specific antigen. Furthermore, we believe that administration of these APCs and/or T cells to a recipient individual may induce immunotolerance in that individual to the antigen. In particular we believe that immunosuppressive cytokines (such as IL-4, IL-10, IL-13, TGF-β and SLIP3 ligand) can be used to upregulate the expression of endogenous Notch ligands in APCs or T cells. The present invention applies these findings to the generation of primed APCs and regulatory T cells using *ex vivo* methods. The resulting primed APCs and/or regulatory T cells may be readministered to the patient to treat or prevent a range of immune disorders resulting from inappropriate T-cell activity, such as auto-immune disease and graft rejection.

Accordingly the present invention provides a method for producing *ex vivo* a T-cell having tolerance to an allergen or antigen which method comprises incubating a T-cell obtained from a human or animal patient with an antigen presenting cell (APC) in the presence of (i) a composition capable of upregulating expression of an endogenous *Notch* ligand in the APC and/or T cell and (ii) the allergen or antigen.

Preferably, the composition comprises a polypeptide selected from Noggin, Chordin, Follistatin, Xnr3, FGF and derivatives, fragments, variants and homologues thereof, and immunosuppressive cytokines, or a combination thereof. More preferably, the composition comprises at least one polypeptide selected from Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants and homologues thereof, together with at least one immunosuppressive cytokine. Particularly preferred examples of

immunosuppressive cytokines for use in the present invention are IL-4, IL-10, IL-13, TGF-β and SLIP3 ligand.

The Notch ligand is preferably selected from Serrate, Delta and homologues thereof, more preferably Serrate and Delta.

The present invention also provides a second method for producing *ex vivo* a T-cell having tolerance to an allergen or antigen which method comprises incubating a T-cell obtained from a human or animal patient with a T-cell produced by the first method of the invention.

T cells produced *ex vivo* by the methods of the invention may be used in suppressing an immune response in a mammal to the allergen or antigen, such as an auto-immune response or allograft rejection.

We have identified substances and combinations of substances capable of upregulating expression of an endogenous Notch ligand in an APC and shown that such substances may be used to produce regulatory T cells capable of suppressing the activity of other T cells.

Accordingly, the present invention also provides the use of a composition capable of upregulating expression of an endogenous *Notch* ligand in an APC and/or T-cell in a method of producing regulatory T cells capable of suppressing the activity of other T cells. Typically, the composition is used *in vitro/ex vivo* rather than *in vivo* and the resulting APCs/T cells subsequently administered to a patient.

Thus the present invention also provides a method of treating a patient suffering from a disease characterised by inappropriate T-cell activity which method comprises administering to the patient a T-cell produced by the methods of the invention.

It is not necessary to incubate the APC and T-cell simultaneously: the APC can be primed first in the presence of the antigen and substance capable of upregulating Notch ligand expression. When such a primed APC is contacted with a T-cell either *in vitro, ex vivo* or *in vivo*, tolerance to the antigen is induced in the T-cell.

Accordingly, the present invention further provides a method for producing an antigen presenting cell (APC) capable of inducing in a T-cell tolerance to an allergen or antigen which method comprises contacting an APC with (i) a composition capable of upregulating expression of an endogenous *Notch* ligand in the APC and (ii) the allergen or antigen. Such APCs may be administered to a patient in a method of immunotherapy. Preferably the method is carried out *ex vivo* using APCs obtained from a human or animal patient suffering from a immune disorder or the recipient of a tissue graft/organ transfer.

Also provided is a method for producing *ex vivo* a T-cell having tolerance to an allergen or antigen which method comprises incubating an APC, produced as described above, with the T-cell.

The present invention further provides a pharmaceutical composition comprising a primed APC and/or T-cell of the invention together with a pharmaceutically acceptable carrier or diluent.

Detailed Description of the Invention

A. Notch ligands

An endogenous Notch ligand in the context of the present invention is a polypeptide encoded by the genome of a mammalian cell that is capable of being expressed by the mammalian cell. In particular the mammalian cell may be a hemapoietic cell such as a T-cell or an antigen presenting cell. The endogenous Notch ligand is typically is capable of binding to a Notch receptor polypeptide present in the membrane of a variety of mammalian cell types, for example hemapoietic stem cells. At least four Notch receptors (Notch-1, Notch-2, Notch-3 and Notch-4) have been identified to date in human cells.

Particular examples of mammalian Notch ligands identified to date include the Delta family, for example Delta (Genbank Accession No. AF003522 - *Homo sapiens*), Delta-3 (Genbank Accession No. AF084576 - *Rattus norvegicus*) and Delta-like 3 (*Mus musculus*),

the Serrate family, for example Serrate-1 and Serrate-2 (WO97/01571, WO96/27610 and WO92/19734), Jagged-1 and Jagged-2 (Genbank Accession No. AF029778 - *Homo sapiens*), and LAG-2. Homology between family members is extensive. For example, human Jagged-2 has 40.6% identity and 58.7% similarity to Serrate.

Further homologues of known mammalian Notch ligands may be identified using standard techniques. By a "homologue" it is meant a gene product that exhibits sequence homology, either amino acid or nucleic acid sequence homology, to any one of the known Notch ligands, for example as mentioned above. Typically, a homologue of a known Notch ligand will be at least 20%, preferably at least 30%, identical at the amino acid level to the corresponding known Notch ligand. Techniques and software for calculating sequence homology between two or more amino acid or nucleic acid sequences are well known in the art (see for example http://www.ncbi.nlm.nih.gov and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.)

Notch ligands identified to date have a diagnostic DSL domain (D. *Delta*, S. *Serrate*, L. Lag2) comprising 20 to 22 amino acids at the amino terminus of the protein and between 3 to 8 EGF-like repeats on the extracellular surface. It is therefore preferred that homologues of Notch ligands also comprise a DSL domain at the N-terminus and between 3 to 8 EGF-like repeats on the extracellular surface.

In addition, suitable homologues will be capable of binding to a Notch receptor. Binding may be assessed by a variety of techniques known in the art including *in vitro* binding assays.

Homologues of Notch ligands can be identified in a number of ways, for example by probing genomic or cDNA libraries with probes comprising all or part of a nucleic acid encoding a Notch ligand under conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C). Alternatively, homologues may also be obtained using degenerate PCR which will generally use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences. The primers will contain one or more

degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

B. Substances capable of upregulating endogenous Notch ligand expression

Substances that may be used to upregulate Notch ligand expression include polypeptides that bind to and reduce or neutralise the activity of bone morphogenetic proteins (BMPs). Binding of extracellular BMPs (Wilson and Hemmati-Brivanlou, 1997, Hemmati-Brivanlou and Melton, 1997) to their receptors leads to down-regulated Delta transcription due to the inhibition of expression of the Achaete/Scute Complex transcription factor. This complex is believed to be directly involved in the regulation of Delta expression. Thus, any substance that inhibits the binding of BMPs to their receptors may be capable of producing an increase in the expression of Notch ligands. Particular examples include Noggin (Valenzuela *et al.*, 1995), Chordin (Sasai *et al.*, 1994), Follistatin (Iemura *et al.*, 1998), Xnr3, and derivatives and variants thereof. Noggin and Chordin bind to BMPs thereby preventing activation of their signalling cascade which leads to decreased Delta transcription.

Furthermore, any substance that upregulates expression of the Achaete/Scute Complex transcription factor may also upregulate Notch ligand expression.

Other suitable substances that may be used to upregulate Notch ligand expression include transforming growth factors such as members of the fibroblast growth factor (FGF) family. The FGF may be a mammalian basic FGF, acidic FGF or another member of the FGF family such as an FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7. Preferably the FGF is not acidic FGF (FGF-1; Zhao *et al.*, 1995). Most preferably, the FGF is a member of the FGF family which acts by stimulating the upregulation of expression of a Serrate polypeptide on APCs. The inventors have shown that members of the FGF family can upregulate Serrate-1 gene expression in APCs.

Immunosuppressive cytokines may also be used to upregulate Notch ligand expression. Examples include members of the TGF-β family such as TGF-β-1 and TGF-β-2, and interleukins such as IL-4, IL-10 and IL-13.

The substance capable of upregulating expression of a Notch ligand may be selected from polypeptides and fragments thereof, linear peptides, cyclic peptides, synthetic and natural compounds including low molecular weight organic or inorganic compounds. The substances capable of upregulating expression of a Notch ligand may be derived from a biological material such as a component of extracellular matrix. Suitable extracellular matrix components are derived from immunologically privileged sites such as the eye. For example aqueous humour or components thereof may be used.

Polypeptide substances such as Noggin, FGFs and TGF-β may be purified from mammalian cells, obtained by recombinant expression in suitable host cells or obtained commercially. Alternatively, nucleic acid constructs encoding the polypeptides may be introduced into APCs and/or T cells by transfection using standard techniques or viral infection/transduction. As a further example, overexpression of Delta or Serrate may be brought about by introduction of a nucleic acid construct capable of activating the endogenous Serrate or Delta gene. In particular, gene activation can be achieved by the use of homologous recombination to insert a heterologous promoter in place of the natural Serrate or Delta promoter in the genome of the APC or T-cell.

It is particularly preferred to use combinations of substances, for example a combination of at least two substances. In a preferred embodiment, an immunosuppressive cytokine is used in combination with another substance capable of upregulating Notch ligand expression. Other examples of preferred combinations include at least one substance capable of upregulating Serrate expression (such as FGF), preferably in an APC, together with at least one substance capable of upregulating Delta expression (such as Noggin or Chordin), preferably in a T-cell. Alternatively, a preferred combination comprises at least one substance which acts via inhibition of binding of BMPs to their receptors together with at least one substance which has a different mode of action.

Preferably, the substance for use in the present invention is capable of upregulating Serrate expression in APCs such as dendritic cells. In particular, the substance may be capable of upregulating Serrate expression but not Delta expression in APCs. Alternatively, the

substance for use in the present invention is capable of upregulating Delta expression in T cells such as $CD4^+$ helper T cells or other cells of the immune system that express Delta (for example in response to stimulation of cell surface receptors). In particular, the substance may be capable of upregulating Delta expression but not Serrate expression in T cells. In a particularly preferred embodiment, the substance is capable of upregulating Notch ligand expression in both T cells and APC, for example Serrate expression in APCs and Delta expression in T cells.

Suitable substances for use according to the present invention may be conveniently identified using a simple screening procedure. In one such assay procedure, T cells or APCs in culture may be contacted with a candidate substance and the effect on expression of an endogenous Notch ligand, such as Delta or Serrate, determined, for example by (i) measuring transcription initiated from the gene encoding the Notch ligand as described in the Examples or by quantitative-reverse transcriptase-polymerase chain reaction (RT-PCR); (ii) detecting Notch ligand protein by techniques such as Western blotting of cell extracts, immunohistochemistry or flow cytometry; and/or (iii) functional assays such as cell adhesion assays.

These procedures may also be used to identify particularly effective combinations of substances for use according to the present invention.

C. Antigen Presenting Cells and T cells

Antigen-presenting cells (APCs) for use in the present invention may be "professional" antigen presenting cells or may be another cell that may be induced to present antigen to T cells. Alternatively a APC precursor may be used which differentiates or is activated under the conditions of culture to produce an APC. The APC may be isolated from a patient, or recipient of the immunotherapy or from a donor individual or another individual. Preferably the APC or precursor is of human origin. If the APC or precursor APC is from a different individual to the T cells, the donor APC may also serve as the source of antigen.

APCs include dendritic cells (DCs) such as interdigitating DCs or follicular DCs, Langerhans cells, PBMCs, macrophages, B-lymphocytes, T-lymphocytes, or other cell types such as epithelial cells, fibroblasts or endothelial cells, activated to express a MHC molecule (Class I or II) on their surfaces. Precursors of APCs include $CD34^+$ cells, monocytes, fibroblasts and endothelial cells. The APCs or precursors may be modified by the culture conditions or may be genetically modified, for instance by transfection of one or more genes encoding proteins which play a role in antigen presentation. Such proteins include MHC molecules (Class I or Class II), CD80, CD86, or CD40. Most preferably DCs or DC-precursors are included as a source of APCs.

The APC or precursor APC may be provided by a cell proliferating in culture such as an established cell line or a primary cell culture. Examples include hybridoma cell lines, L-cells and human fibroblasts such as MRC-5. Cell lines may conveniently be used in the screening procedures described above.

Dendritic cells (DCs) can be isolated/prepared by a number of means, for example they can either be purified directly from peripheral blood, or generated from $CD34^+$ precursor cells for example after mobilisation into peripheral blood by treatment with GM-CSF, or directly from bone marrow. From peripheral blood, adherent precursors can be treated with a GM-CSF/IL-4 mixture (Inaba *et al.*, 1992), or from bone marrow, non-adherent $CD34^+$ cells can be treated with GM-CSF and TNF-α (Caux *et al.*, 1992). DCs can also be routinely prepared from the peripheral blood of human volunteers, similarly to the method of Sallusto and Lanzavecchia (1994) using purified peripheral blood mononucleocytes (PBMCs) and treating 2 hour adherent cells with GM-CSF and IL-4. If required, these may be depleted of $CD19^+$ B cells and $CD3^+$, $CD2^+$ T cells using magnetic beads (see Coffin *et al.*, 1998). Culture conditions may include other cytokines such as GM-CSF or IL-4 for the maintenance and, or activity of the dendritic cells or other antigen presenting cells.

Where T cells are to be used in *ex vivo* methods of inducing immunotolerance, the T cells for use in the invention are typically isolated from an individual suffering from a disease of the immune system or a recipient for a transplant operation or from a related or unrelated donor individual. T cells may be obtained from blood or another source (such as lymph

nodes, spleen, or bone marrow) and may be enriched or purified by standard procedures. The T cells may be used in combination with other immune cells, obtained from the same or a different individual. Alternatively whole blood may be used or leukocyte enriched blood or purified white blood cells as a source of T cells and other cell types. It is particularly preferred to use helper T cells ($CD4^+$). Alternatively other T cells such as $CD8^+$ cells may be used.

Where T cells are to be used in *in vitro* screening procedures, it may be convenient to use cell lines such as T-cell hybridomas.

E. Antigens

The antigen may be a peptide, polypeptide, carbohydrate, protein, glycoprotein, or more complex material containing multiple antigenic epitopes such as a protein complex, cell-membrane preparation, whole cells (viable or non-viable cells), bacterial cells or virus/viral component. In particular, it is preferred to use antigens known to be associated with auto-immune diseases such as myelin basic protein (associated with multiple sclerosis), collagen (associated with rheumatoid arthritis), and insulin (diabetes), or antigens associated with rejection of non-self tissue such as MHC antigens. Where primed APCs/ T cells of the present invention are to be used in tissue transplantation procedures, antigens will be obtained from the tissue donor.

The antigen or allergen moiety may be, for example, a synthetic MHC-peptide complex i.e. a fragment of the MHC molecule bearing the antigen groove bearing an element of the antigen. Such complexes have been described in Altman *et al.*, 1996.

F. Preparation of Primed APCs and T cells

1. Preparation of Primed APCs *ex vivo* in the absence of T cells

APCs as described above are cultured in a suitable culture medium such as DMEM or other defined media, optionally in the presence of fetal calf serum. Cytokines, if present, are

typically added at up to 1000 U/ml. Optimum concentrations may be determined by titration. One or more substances capable of upregulating Notch ligand expression are then typically added to the culture medium together with the antigen of interest. The antigen may be added before, after or at substantially the same time as the substance(s). Cells are typically incubated with the substance(s) and antigen for at least one hour, preferably at least 3 hours, at 37°C. If required, a small aliquot of cells may be tested for upregulation of Notch ligand expression as described above. Alternatively, cell activity may be measured by the inhibition of T-cell proliferation as described in WO98/20142. APCs transfected with a nucleic acid construct directing the expression of, for example Serrate, may be used as a control.

As discussed above, polypeptide substances may be administered to APCs by introducing nucleic acid constructs/viral vectors encoding the polypeptide into cells under conditions that allow for expression of the polypeptide in the APC. Similarly, nucleic acid constructs encoding antigens may be introduced into the APCs by transfection, viral infection or viral transduction.

The resulting APCs that express increased levels of a Notch ligand and are presenting antigen on their cell surface complexed with MHC are now ready for use. For example, they may be prepared for administration to a patient or incubated with T cells *in vitro* (*ex vivo*) to induce immunotolerance in the T cells as described in WO98/20142.

2. <u>Preparation of Regulatory T cells *ex vivo*</u>

The techniques employed are essentially identical to that described for APCs alone except that T cells are generally co-cultured with the APCs. However, it may be preferred to prepare primed APCs first and then incubate them with T cells. For example, once the primed APCs have been prepared, they may be pelleted and washed with PBS before being resuspended in fresh culture medium. This has the advantage that if, for example, it is desired to treat the T cells with a different substance(s) capable of upregulating Notch ligand expression and/or cytokine to that used with the APC, then the T-cell will not be brought into contact with the different substance(s) used to upregulate Notch ligand expression in the APC. Alternatively, the T-cell may be incubated with the substance(s)/cytokine first to induce Notch ligand

expression, washed, resuspended and then incubated with the primed APC in the absence of both the substance(s) used to upregulate APC Notch ligand expression and the substance(s) used to upregulate Notch ligand expression in the T-cell. Once primed APCs have been prepared, it is not always necessary to administer any substances to the T-cell since the primed APC is itself capable of inducing immunotolerance leading to increased Notch ligand expression in the T-cell, presumably via Notch/Notch ligand interactions between the primed APC and T-cell.

Incubations will typically be for at least 1 hour, preferably at least 3 or 6 hours, in suitable culture medium at 37°C. The progress of induction of Notch ligand expression may be determined for a small aliquot of cells using the methods described above. T cells transfected with a nucleic acid construct directing the expression of, for example Delta, may be used as a control. Induction of immunotolerance may be determined by subsequently challenging T cells with antigen and measuring IL-2 production compared with control cells not exposed to APCs.

Primed T cells may also be used to induce immunotolerance in other T cells in the absence of APCs using similar culture techniques and incubation times. Generally, the addition of substances capable of upregulating Notch ligand expression is not required at this stage but they may be added if desired, together with immunosuppressive cytokines.

G. Therapeutic Uses

We have shown that APCs and T cells expressing Notch ligands are capable of efficiently transferring infectious tolerance to the chosen antigen or antigens when transferred into the patient for the treatment of a disease characterised by inappropriate T-cell activity, such as Th1 or Th2 cell activity. The APCs and/or T cells may thus be used to treat an ongoing immune response (such as an allergic condition or an autoimmune disease) or may be used to generate tolerance in an immunologically naïve individual (such as a recipient for a transplant operation). Thus the APCs and/or T cells of the present invention may be used in therapeutic methods for both treating and preventing diseases characterised by

inappropriate T-cell activity in animals and humans. The APCs and/or T cells may be used to confer tolerance to a single antigen or to multiple antigens.

Typically, APCs and/or T cells are obtained from the patient or donor and primed as described above before being returned to the patient (*ex vivo* therapy).

Particular conditions that may be treated or prevented include multiple sclerosis, rheumatoid arthritis, diabetes, allergies, asthma, and graft rejection. The present invention may also be used in organ transplantation or bone marrow transplantation.

H. Administration

Primed APCs/T cells of the present invention for use in immunotherapy are typically formulated for administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, intra-peritoneal, injection, intranasal inhalation, lung inhalation, intradermal, intra-articular, intrathecal, or via the alimentary tract (for example, via the Peyers patches).

Cells and pharmaceutical comprising cells of the invention are typically administered to the patient by intramuscular, intraperitoneal or intravenous injection, or by direct injection into the lymph nodes of the patient, preferably by direct injection into the lymph nodes. Typically from 10^4 to 10^8 treated cells, preferably from 10^5 to 10^7 cells, more preferably about 10^6 cells are administered to the patient.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient depending on, for example, the age, weight and condition of the patient.

The present invention will now be described by way of examples which are intended to be illustrative only and non-limiting.

EXAMPLES

Materials and Methods

Construction of Retroviruses expressing Delta or Serrate

A cDNA encoding human Serrate-1 is inserted as a *Bam*HI – *Eco*RI fragment into the retroviral vector pBABEneo (Morgenstern and Land, 1990) using standard techniques, such that the cDNA is expressed from the retroviral promoter element. Transducing vector particles can be produced by transfection of this construct into a suitable amphotropic packaging cell line such as PA317 (ATCC catalogue number CRL-9078), FLYA13 or FLYRD114 (Cosset *et al.*, 1995). Alternatively a permanent cell line containing the vector genome and gag-pol gene is constructed by transfection of Serrate-pBABE vector into PA327 cells. Production of replication – deficient pseudotyped vector particles is initiated by transient transfection with a plasmid encoding the G-protein from Vesicular Stomatitis Virus (VSV) under the control of the hCMV major immediate early promoter-enhancer.

A retroviral vector expressing mouse Delta-1 is prepared in a similar fashion.

Preparation of Dendritic Cells from Mouse Spleen

Single cell suspensions are prepared from the spleens of mice. Rapidly adhering cells are isolated by culture for 2-3 h at 37°C in plastic tissue culture flasks. Non-adherent cells are removed by extensive washing and 50 ng/ml mouse GM-CSF in culture medium is added for 24 h. Culture medium is RPMI 1640 with 2% FCS, 50 mM 2-mercaptoethanol, and optionally 0.02 mg/ml Penicillin and Streptomycin.

Preparation of T cells from Mouse

T cells are purified from blood or lymph nodes by positive selection on magnetic antibody-coated beads specific for particular cell types (MACS columns) using methods provided by the manufacturer (Miltenyi Biotech) as follows.

Lymph nodes are removed and single cell suspensions prepared in tissue culture medium (10^8 cells in 0.4 ml RPMI 1640 with 10% FCS). Cells are incubated at 4°C for 15 min, passed over the MACS separation column, washed and collected. CD4-positive cells are enriched by negative selection on anti-CD8 antibody-coated magnetic beads.

Determination of Serrate and Delta expression.

After various incubation times, cells (1.5×10^6) are harvested, pelleted and frozen. RNA is prepared from cell pellets by homogenisation in guanidium thiocyanate solution followed by CsCl density centrifugation. 1 μg RNA is converted into cDNA using an oligo dT primer. Of the resultant cDNA, 1/20th was used in PCR (40 cycles) using primers specific for the human delta homologue or the human serrate homologue.

RT-PCR is performed using an Access RT-PCR kit (Promega). 50 ng RNA is used in each reaction together with Serrate-1 gene specific oligonucleotide primers (50 pmol) under conditions according to the manufacturer's instructions (T_m for the Serrate oligonucleotides is 58°C).

The sequence of the "forward" Serrate-1 primer is:
5'-GGCTGGGAAGGAACAACCTG-3'

The Serrate- "reverse" primer is:
5'-GGTAGCCATTGATCTCATCCAC-3'

Primers specific for Delta are:
5'-GATTCTCCTGATGACCTCGC-3'
5'-GTGTTCGTCACACACGAAGC-3'

PCR samples were analysed by gel electrophoresis.

Example 1 - Assays to determine to identify substances that upregulate Notch ligand expression.

Dendritic cells (DCs) are the primary antigen presenting cell in the immune system and are critical for stimulating T-cell responses. DCs are obtained from the spleen of mice and transferred to flasks containing tissue culture medium (RPMI 1640 with 10% fetal calf serum added). Cytokines (eg IL-4 and GM-CSF) are added as appropriate.

Cells are then transferred into 12-well tissue culture trays. To each well is added a different candidate upregulator of Notch ligand expression. Delta and Serrate expression is monitored at various time points by removing an aliquot of cells and determining induction of Delta and Serrate expression by PCR.

Similar procedures are also carried out using a T-cell hybridoma cell line and T cells obtained from mice as described in the materials and methods section.

Example 2 - Preparation of Primed Dendritic Cells

DCs are obtained from the spleen of mice as in Example 1 and divided into two cultures. The first culture is transfected with a retrovirus allowing expression of the full length Serrate-1 protein to serve as a positive control. The first culture is then pulsed with the HDM peptide p110-131 for 3 hours at 37°C. The second culture is split up into several tissue culture plate wells and to each well is added a different upregulator of Notch ligand expression identified in Example 1. These wells are then also pulsed with the HDM peptide p110-131 for 3 hours at 37°C

The DCs are then washed and used to immunise naive mice subcutaneously using 10^5 cells/mouse. After 7 days the draining LNCs are recovered and restimulated in culture with peptide at $4x10^5$ cells/well. Since the mice were only immunised with peptide-pulsed DCs this gives us a measure of the ability of these cells to prime an immune response.

Example 3 - Upregulation of Serrate expression in antigen presenting cells prevents T-cell responses.

An influenza-reactive human T-cell clone HA1.7 is mixed with peptide HA306-318 (1.0 μg/ml) in the presence of L cells expressing HLA-DRB1*0101(as antigen presenting cells), using $2x10^4$ of each cell type. The L cells have been preincubated with one or more substances identified as being capable of upregulating Serrate expression in APCs for 6 hours. The proliferative response is measured after 72 hours following addition of tritiated thymidine for the last 8 hours of culture.

Example 4 - Serrate upregulated antigen presenting cells induce regulatory T cells that can block the response of normal T cells.

An influenza-reactive human T-cell clone HA1.7 is mixed with peptide HA306-318 and L cells (expressing DRB1*0101 as antigen presenting cells) in the presence of 2% IL-2. The L cells have been preincubated with one or more substances identified as being capable of upregulating Serrate expression in APCs for 6 hours. After 7 days in culture, the HA1.7 cells were harvested, washed and irradiated before being mixed with fresh HA1.7 (using $2x10^4$ each population). Cells are cultured for a further 2 days before being stimulated with peptide (1.0 μg/ml) + normal APCs (DRB1*0101 PBMCs). The proliferative response is measured after 72 hours following addition of tritiated thymidine for the last 8 hours of culture.

The results show the ability of cells tolerised by upregulation of Serrate to pass on their tolerance to a naive cell population (infectious/bystander tolerance).

Example 5 - Preparation of regulatory T cells *ex vivo* using primed APCs

Primed dendritic cells are produced using the same method as in Example 2. These cells are then washed, pelleted and resuspended in fresh culture medium. T cells obtained from the mouse host are then incubated with the primed dendritic cells for up to 6 hours. Aliquots of cells are taken at regular intervals and Delta and Serrate expression measured.

Helper T cells are separated from the other cells using magnetic beads specific for CD4 prior to pelleting and RNA extraction.

Induction of immunotolerance in the T cells is also measured in a functional assay. HDM peptide p110-131 added to the cell culture and the cells cultured for 24 hours. Supernatant fluids are then collected and assayed for IL-2 (a major T-cell growth factor) content.

Example 6 - Delta-upregulated primed T cells are able to inhibit the development of immunity to peptide 110-131 antigen in animals.

1×10^7 primed regulatory T cells generated by the method described in Example 4 are injected into C57 BL mice i.p. The mice are also immunised with 50 μg Der p1 emulsified in Complete Freunds Adjuvant (CFA) sub-cutaneously. After 7 days the draining lymph node cells are collected and cultured at 4×10^5 cells/well with Der p1 (10 μg /ml) or peptide 110-131 of Der p1 (10 μg /ml). Cultures are incubated at 37°C for 72 hours and tritiated thymidine added for the final 8 hours of culture.

The results show that the primed regulatory T cells inhibit the development of an immune response to the Der p1 antigen in the immunised mouse.

Example 7 – Treatment of patients undergoing Bone Marrow Transplantation

Donor individuals for the bone marrow transplantation procedure are selected from an appropriate category (live related; MHC-matched un-related or unmatched); DCs are isolated from the donor by a suitable method (eg as described in US-A-5789148) between 14 days prior to, and 3 days after, transplantation. DCs are maintained in culture in tissue culture medium eg RPMI-1640 supplemented with up to 10% autologous or ABO human serum). Inducers of Notch-ligand expression are added for the appropriate time (between 3 h and 2 days). Cytokines are also added as required (eg IL-4 and GM-CSF).

DCs may be similarly prepared from the transplant recipient if required.

Lymphocytes are obtained by an appropriate method (e.g. according to the procedures described in US-A-4663058) from the donor and/or recipient. T cells may be enriched by standard methods including antibody-mediated separation. Cells are cultured in RPMI-1640 with serum (autologous or ABO human serum) together with DCs.

T cells and DCs are then transferred to the transplant recipient by infusion at a suitable time, between 14 days before and 3 days after transplantation.

Other modifications of the present invention will be apparent to those skilled in the present art.

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CLAIMS

1. A method for producing *ex vivo* a T-cell having tolerance to an allergen or antigen which method comprises incubating a T-cell obtained from a human or animal patient with an antigen presenting cell (APC) in the presence of (i) a composition capable of upregulating expression of an endogenous *Notch* ligand in the APC and/or T cell and (ii) the allergen or antigen.

2. A method according to claim 1 wherein the composition comprises a polypeptide selected from Noggin, Chordin, Follistatin, Xnr3, FGF and derivatives, fragments, variants and homologues thereof, and immunosuppressive cytokines, or a combination thereof.

3. A method according to claim 2 wherein the composition comprises at least one polypeptide selected from Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants and homologues thereof, together with at least one immunosuppressive cytokine.

4. A method according to claim 2 or 3 wherein the immunosuppressive cytokine is selected from IL-4, IL-10 and TGF-β.

5. A method according to any one of the preceding claims wherein the Notch ligand is selected from Serrate, Delta and homologues thereof.

6. A method according to any one of the preceding claims wherein the APC is a dendritic cell.

7. A method for producing *ex vivo* a T-cell having tolerance to an allergen or antigen which method comprises incubating a T-cell obtained from a human or animal patient with a T-cell produced by the method of any one of the preceding claims.

8. Use of a T-cell produced by the method of any one of the preceding claims in suppressing an immune response in a mammal to the allergen or antigen.

9. Use of a composition capable of upregulating expression of an endogenous Notch ligand in an APC in a method of producing regulatory T cells capable of suppressing the activity of other T cells.

10. Use according to claim 9 wherein the composition is as defined in any one of claims 2 to 4.

11. Use according to claim 9 or 10 wherein the Notch ligand is selected from Serrate, Delta and homologues thereof.

12. Use according to any one of claims 9 to 11 wherein the APC is a dendritic cell.

13. A method of treating a patient suffering from a disease characterised by inappropriate T-cell activity which method comprises administering to the patient a T-cell produced by the method of any one of claims 1 to 7.

14. A method for producing an antigen presenting cell (APC) capable of inducing in a T-cell tolerance to an allergen or antigen which method comprises contacting an APC with (i) a composition capable of upregulating expression of an endogenous Notch ligand in the APC and (ii) the allergen or antigen.

15. A method for producing a T-cell having tolerance to an allergen or antigen which method comprises incubating an APC produced by the method of claim 14 with the T-cell.

ABSTRACT

METHODS OF IMMUNOSUPPRESSION

A method for producing a T-cell having tolerance to an allergen or antigen which method comprises incubating the T-cell with an antigen presenting cell (APC) in the presence of (i) a composition capable of upregulating expression of an endogenous Notch ligand in the APC and (ii) the allergen or antigen is provided.